Kidney International, Vol. 62 (2002), pp. 2097–2100

ION CHANNELS – MEMBRANE TRANSPORT – INTEGRATIVE PHYSIOLOGY

Large-scale preparation of sodium-potassium ATPase from kidney outer medulla

Irena Klodos, Mikael Esmann, and Robert L. Post

Department of Biophysics, Aarhus University, Aarhus, Denmark, and Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania, USA

Large-scale preparation of sodium-potassium ATPase from kidney outer medulla.

Background. Large amounts of Na,K-ATPase are needed for studies involving protein chemistry. Preparation of Na,K-ATPase from kidney by the widely used, rapid procedure of Jørgensen (*Biochim Biophys Acta* 356:36-52, 1974; *Methods Enzymol* 156:29-43, 1988) includes labor-intensive dissection of tissue from the outer medulla and centrifugation into a step gradient of sucrose solution.

Methods. In a large-scale modification presented here, tissue was dissected with a surgical instrument, a rongeur, and centrifugation was simply a five times repeated differential centrifugation. The procedure took seven days and 68 person-hours of work.

Results. The yield of activity from 26 kg of whole kidneys was 6600 units (μmol Pi/min) in one preparation. The amount of protein was 240 mg and the specific activity was 28 μmol Pi/min per mg protein.

Conclusions. There is a significant saving of labor to obtain a product with a specific activity similar to that commonly obtained. The microsomal fraction may be useful for preparing other membrane proteins from the outer medulla.

Sodium-potassium ATPase (Na,K-ATPase) generates concentration gradients of sodium and potassium ions across the plasma membrane of animal cells. The gradients provide the source of energy for membrane transport of substances such as sugars, amino acids and metabolites and for electrical activity of nerve and muscle. More than 45% of energy usage of kidney and brain is consumed by Na,K-ATPase [1]. Sources of this enzyme for experimental work are organs that transport much sodium and potassium. The outer medulla of mammalian kidney is an excellent source and Jørgensen's rapid procedure is frequently used [2, 3]. However, dissection of the kidney described there is labor-intensive. The success of Jørgensen's procedure depends critically on dissection of tissue from the red outer medulla (inner stripe of the outer medullary zone). In this method kidneys are cut in longitudinal sections 0.5 to 1 cm thick. Dissection is performed on an ice-cold stainless steel plate covered with moistened filter paper. The dissecting knife is guided by the sense of vision. This is "sharp dissection," which is distinct from "blunt dissection" in the terminology of surgeons. The dissection requires much time. We describe a simpler method of dissection that yields microsomes rich in Na,K-ATPase. The method is suitable for preparation of large amounts of enzyme needed for work involving protein chemistry. The microsomes may be useful as a source for other membrane proteins in the outer medulla.

Key words: sodium, potassium, adenosinetriphosphatase, media preparation, protein chemistry, microsomes, Jørgensen's procedure.

Received for publication September 4, 2001
and in revised form June 13, 2002
Accepted for publication July 3, 2002

METHODS

Dissection of outer medulla (Day 1)

Pig kidneys obtained fresh from a slaughterhouse were transported to the laboratory wrapped in plastic film and packed in ice. Kidneys visibly different from the majority were discarded. Each kidney was sliced from one pole to the other with the plane of the cut passing through the hilus. Regions of outer medulla were identified by their dark red color [3, 4]. No attempt was made to select the inner stripe of the outer medulla.

Pieces of outer medulla were bitten out with a special surgical instrument, a rongeur (for an image see Rongeur No. 26-192, about $240, at *www.ssrsurgical.com/pg414.htm*). A rongeur is used by orthopedic surgeons to bite off pieces of bone. The handles are like a pair of scissors but instead of blades the instrument has a pair of small cups (diameter 6 or 8 mm) with sharp edges. When these cups are pressed into soft tissue and are brought together, they bite out a piece of the tissue. In the case of outer medulla the bite is selective. It is selective because the outer medulla is separated anatomically from underlying cortex of the kidney by a layer of dense fibrous connective tissue. Thus, a moderate force pushing

the rongeur into the outer medulla brings the open cups to the limit of outer medulla tissue. When the cups are forced together, the outer medulla is captured by the cups and the dense fibrous layer and cortex are left behind. The dissection is guided partly by the sense of touch. This is "blunt dissection." (We used two curved rongeurs from Martin Medizin-Technik, *www.martin-med.com*; 8 mm diameter, Martin 23-847-17, and 6 mm diameter, Martin 23-819-18. The smaller size fits a smaller hand.) In one day's work the yield of outer medulla tissue was 336 g from 13 kg of kidneys. About 12 person-hours are required for the dissection.

Disruption of tissue

The outer medulla tissue was suspended in ice-cold ISE-buffer consisting of imidazole 25 mmol/L, sucrose 250 mmol/L, and ethylenediaminetetraacetic acid (EDTA) 1 mmol/L (pH 7.4 at 20°C) to a final volume of about 500 mL. To disperse the outer medulla and remove residual connective tissue a tissue press was used. Fifty milliliter portions of the suspension were forced through a plate perforated throughout with 2.0 mm diameter holes. Each portion contained about 34 grams of tissue. The process was repeated through a plate with 1.4 mm diameter holes. The sieved suspension was stored overnight at 4°C. This stage of the preparation required two person-hours of work. (We constructed our tissue press. It is a bakelite cylinder 6.5 cm high and 3.5 cm in inner diameter. At the bottom is one of two replaceable stainless steel plates perforated throughout with holes 2.0 or 1.4 mm in diameter. The piston is a bakelite rod 3.5 cm in diameter. We have not tested a tissue press from BioSpec Products, Inc.; *www.biospec.com/Brochures/TissPres/TisPres.html*.)

Preparation of microsomes by differential centrifugation (day 2)

To the 500 mL of sieved suspension was added 500 mL of cold ISE-buffer with stirring. The mixture was homogenized ice-cold in 80 mL portions in a 60-mL pestle homogenizer with a teflon piston at 2000 revolutions per minute, three times down and up. Then two liters more of ISE were added with stirring. The mixture was centrifuged at 3700 × *g*-average for 20 minutes at 5°C in a rotor holding 1.5 L (Sorvall GSA rotor at 6000 rpm). The supernatant was kept on ice while the pellets were resuspended in ISE-buffer to a volume of 1 liter and the homogenization, dilution and centrifugation were repeated as before. This pellet was discarded and the combined supernatants were centrifuged at 7400 × *g*-average (8500 rpm) otherwise as stated earlier. The pellet was again discarded. The supernatant was centrifuged at 38,000 × *g*-average for 40 minutes at 5°C in a 400 mL angle rotor (Sorvall SS-34 or Beckman Ti45 at 20,000 rpm). This supernatant was discarded and the 14 pellets were suspended in 150 mL of ISE-buffer and homogenized as described above with five strokes down and up. Then, 50 mL more of ISE-buffer was added and an aliquot of 1 mL was separated. The aliquot was used for a titration with sodium dodecyl sulfate (SDS) on day 5. The microsomal fraction and the aliquot were stored at −20°C. This stage of the preparation required 12 person-hours of work.

Na,K-ATPase activity was measured as ouabain-sensitive release of inorganic phosphate from ATP in the presence of Mg^{2+}, Na^+, and K^+ [5]. At this stage of preparation the specific activity after activation by detergent, as described later, was 6.0 U/mg protein. The yield was 6720 units (μmol Pi/min) of activity in 1120 mg (5.6 mg/mL). Protein was measured by the method of Lowry et al [6] against bovine serum albumin as a standard without correction for color factor [7, 8]. The activity without detergent activation was 1.5 U/mg, indicating that 3/4 of the enzyme was in tight vesicles [9]. Enzyme in tight vesicles was inactive because substrates in the small volume of the interior reach a rate-limiting concentration after very little activity has taken place. In the case of right-side-out vesicles intravesicular ATP was depleted after the enzyme had turned over only a few times; in the case of inside-out vesicles intravesicular K^+ was depleted correspondingly since K^+ is a stoichiometric substrate for transport. Gentle treatment with detergent made the vesicles permeable to substrates in the medium.

On Days 3 and 4, the procedure described so far was repeated producing a second 200 mL of frozen microsomes for 14 more person-hours of work.

Titration with SDS (day 5)

Treatment with SDS removed extraneous proteins while leaving the Na,K-ATPase membrane bound [3]. The amount of SDS to add was estimated by titration of aliquots of the enzyme as follows. Microsomes were diluted to 4.6 mg/mL by addition of ISE-buffer. To 0.08 mL of this diluted microsomal suspension was added 0.02 mL of ISE-buffer containing a variable amount of SDS. After incubation for 60 minutes at room temperature 0.9 mL of ISE was added, and 0.05 mL of this was transferred to 0.9 mL of a test solution containing NaCl 130 mmol/L, KCl 20 mmol/L, $MgCl_2$ 4 mmol/L and ATP 3 mmol/L in histidine buffer 30 mmol/L (pH 7.4 at 37°C). After three minutes at 37°C, trichloroacetic acid was added and inorganic phosphate liberated was measured [5]. The end-point of the titration was at the smallest addition of SDS that decreased the activity. In one preparation the activity at 0.9 mg SDS/mL was 96% of that at 0.8 mg/mL and so 0.9 mg SDS/mL was used for purification. The ratio of SDS to protein was 0.24 mg SDS/mg protein. This step requires about five person-hours for separate titrations of two microsomal fractions.

Table 1. Comparison of blunt and sharp dissection procedures for the preparation of Na,K-ATPase

Stage	Amount *g*		Activity *units*		Person-hours	
	Blunt	Sharp	Blunt	Sharp	Blunt	Sharp
Whole kidneys *wet weight*	26,000	28,000			[a]	[a]
Outer medulla *wet weight*	672	1300			24	60
Microsomes *protein*	2.24	1.80[b]	13,440	12,600	28	66
Purified Na,K-ATPase *protein*	0.24	0.18[b]	6600	3160	17	[c]
Total time to prepare microsomes					52	126

The methods for the blunt procedure are those of this study. The methods for the sharp procedure are those of Taniguchi et al [11].
[a] Time required to obtain the kidneys is not included
[b] Estimated by the Bradford method [12]
[c] Data not collected

Treatment with SDS (day 6)

The two frozen microsome preparations were thawed in a water bath at room temperature and each preparation was treated with SDS separately. Each microsomal fraction was diluted to 4.6 mg/mL by the addition of 43 mL of ISE-buffer. One-fourth volume (61 mL) of ISE-buffer containing the estimated amount of SDS (274 mg in this case) was added. (ATP was not included in the treatment solution [2, 3] because it confers no advantage at the end point used here.) The two fractions were combined after the addition of SDS and the suspension (about 600 mL) was allowed to stand in the presence of SDS overnight at 20°C. One person-hour was required for this treatment.

Centrifugation (day 7)

The treated microsomal suspension is divided into two portions and centrifuged at 127,000 × *g*-average for 50 minutes at 10°C (Beckman Ti45 rotor at 40,000 rpm). The supernatant was discarded and the pellets combined and resuspended in 420 mL of ISE-buffer. Centrifugation and resuspension were repeated four times. The volume of the last resuspension was adjusted to a protein concentration of about 4 mg/mL. This stage requires seven person-hours of work. The final assay of enzyme activity and amount of protein requires four person-hours of work.

Schedule

To perform the large scale preparation, the procedures done on days 1 and 2 are consecutive. Days 3 and 4 are consecutive but not necessarily in the same week as days 1 and 2. Day 5 can be up to three months later. Days 6 and 7 are consecutive.

RESULTS

The yield of activity from 26 kg of whole kidneys providing 670 g of outer medulla was 6600 units (μmol Pi/min) in one preparation. The amount of protein was 240 mg and the specific activity was 28 μmol Pi/min per mg protein. Nucleotide binding capacity [10] was 3.0 nmol/mg and gave a turnover number (molar activity) of 9000 per minute. The ouabain-insensitive ATPase activity was 0.5 to 1% of Na,K-ATPase activity. The whole procedure required about 69 person-hours of work. The yield of outer medulla per kidney was lower than that obtained by sharp dissection but the yield per person hour showed a saving of effort (Table 1). Ten preparations had specific activities of 28.8 ± 2.3 μmol Pi/min per mg protein for Na,K-ATPase activity and 4.4 ± 0.4 μmol Pi/min per mg protein for K-dependent *p*-nitrophenylphosphatase activity (pNPPase). The preparation consisted almost entirely of alpha and beta subunits of Na,K-ATPase (Fig. 1).

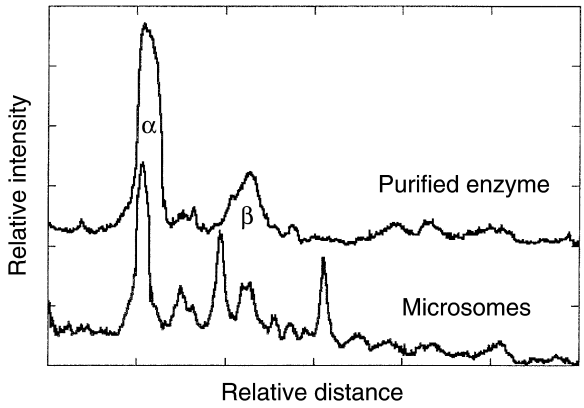

Fig. 1. Purity of a large-scale Na,K-ATPase preparation. Scan of preparations analyzed by polyacrylamide gel electrophoresis [2, 13]. Thirty micrograms of protein were applied to each lane. The lower trace is of the microsomes; the upper trace is of the purified enzyme, showing the alpha (α) and beta (β) subunits. Both traces are offset from the baseline to avoid overlap.

DISCUSSION

The quality of a preparation can be evaluated by several criteria. The first criterion is an absence of contaminating proteins. Contaminating proteins can be detected by polyacrylamide gel electrophoresis, except for those proteins that run with the alpha and beta subunits of

Na,K-ATPase [2]. These are probably not present in significant amounts because isoelectric focusing of isolated alpha and beta subunits did not reveal other proteins [14]. Polyacrylamide gel electrophoresis of the large-scale preparation presented here showed little evidence of contaminating proteins (Fig. 1).

Another criterion is specific activity, which is the ratio of activity under standard conditions to the amount of protein. Standard conditions are saturating concentrations of ATP, Mg^{2+}, Na^+, and K^+ at 37°C. The amount of protein varies somewhat according to the method of measurement [15]. Specific activity can range from 25 μmol/min/mg [2] to 50 μmol/min/mg [3]. Specific activity can be low not only because of contaminating protein, but also because of physiological down regulation or mild injury. Mild injury impairs some partial activities more than others. Nørby and Jensen found that different activities were impaired at different doses of ionizing radiation [16]. Harris, Swanson and Stahl found that ouabain-binding was more stable than (Na^+,K^+)-ATPase activity to treatment with agents known to affect membrane integrity [17]. Finally, binding of Rb^+ persists even after extensive proteolytic digestion that produces complete loss of activity [18]. Thus, the ratio of activity to ligand binding is a measure of enzyme quality. The ratio of specific activity to ligand binding capacity is the turnover number, also known as molecular activity. The turnover number is independent of estimation of protein. Values from 7700 min^{-1} in enzyme from kidney [3, 8] to 9700 min^{-1} in enzyme from duck salt gland [15] have been reported. There is also a further criterion, the ratio of Na,K-ATPase activity to pNPPase activity, since pNPPase activity is less sensitive to damage to the enzyme than is Na,K-ATPase activity, although it is more sensitive than ligand binding capacity [16]. Values of the ratio of 5.3 in kidney [8] and 7.3 in rectal gland [19] have been reported. Further discussion can be found in [5].

ACKNOWLEDGMENTS

This work was supported by The Danish Medical Research Council (grant number 52-00-0914). We are grateful to Ms. Angielina Damgaard and Ms. Birthe Bjerring Jensen for excellent technical assistance. We are grateful to Dr. Kazuya Taniguchi for providing data for the sharp method of preparation of Na,K-ATPase.

Reprint requests to Mikael Esmann, D.Sc., Department of Biophysics, University of Aarhus, Ole Worms Allé 185, DK-8000 Aarhus C, Denmark.
E-mail: me@biophys.au.dk

REFERENCES

1. Cornelius F: The sodium pump, in *Biomembranes. ATPases* (vol 5), edited by Lee AG, Greenwich, JAI Press Inc., 1996, pp 133–184
2. Jørgensen PL: Purification and characterization of (Na^+ + K^+)-ATPase. III. Purification from the outer medulla of mammalian kidney after selective removal of membrane components by sodium dodecylsulphate. *Biochim Biophys Acta* 356:36–52, 1974
3. Jørgensen PL: Purification of Na^+,K^+-ATPase: Enzyme sources, preparative problems, and preparation from mammalian kidney. *Methods Enzymol* 156:29–43, 1988
4. Kelly DE, Wood RL, Enders AC: *Bailey's Textbook of Microscopic Anatomy* (18 ed). Baltimore, Williams & Wilkins, 1984, p 646
5. Esmann M: ATPase and phosphatase activity of Na^+,K^+-ATPase. Molar and specific activity, protein determination. *Methods Enzymol* 156:105–119, 1988
6. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275, 1951
7. Chetverin AB: Evidence for a diprotomeric structure of Na,K-ATPase. Accurate determination of protein concentration and quantitative endgroup analysis. *FEBS Lett* 196:121–125, 1986
8. Jensen J, Nørby JG, Ottolenghi P: Binding of sodium and potassium to the sodium pump of pig kidney evaluated from nucleotide binding behaviour. *J Physiol (Lond)* 346:219–241, 1984
9. Forbush B III: Characterization of right-side-out membrane vesicles rich in (Na,K)-ATPase and isolated from dog kidney outer medulla. *J Biol Chem* 257:12678–12684, 1982
10. Fedosova NU, Champeil P, Esmann M: Nucleotide binding to Na,K-ATPase: The role of electrostatic interactions. *Biochemistry* 41:1267–1273, 2002
11. Taniguchi K, Suzuki K, Iida S: Conformational change accompanying transition of ADP-sensitive phosphoenzyme to potassium-sensitive phosphoenzyme of (Na^+,K^+)-ATPase modified with *N*-[*p*-(2-benzimidazolyl)phenyl]maleimide. *J Biol Chem* 257:10659–10667, 1982
12. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976
13. Schagger H, Von Jagow G: Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 166:368–379, 1987
14. Yamaguchi M, Post RL: Isoelectric focussing of the catalytic subunit of (Na,K)-ATPase from pig kidney. *J Biol Chem* 258:5260–5268, 1983
15. Martin DW, Sachs JR: Preparation of Na^+,K^+-ATPase with near maximal specific activity and phosphorylation capacity. Evidence that the reaction mechanism involves all of the sites. *Biochemistry* 38:7485–7497, 1999
16. Nørby JG, Jensen J: A model for the stepwise radiation inactivation of the α_2-dimer of Na,K-ATPase. *J Biol Chem* 264:19548–19558, 1989
17. Harris WE, Swanson PD, Stahl WL: Ouabain binding sites and the (Na^+,K^+)-ATPase of brain microsomal membranes. *Biochim Biophys Acta* 298:680–689, 1973
18. Capasso JM, Hoving S, Tal DM, *et al*: Extensive digestion of Na^+,K^+-ATPase by specific and nonspecific proteases with preservation of cation occlusion sites. *J Biol Chem* 267:1150–1158, 1992
19. Skou JC, Esmann M: Preparation of membrane Na^+,K^+-ATPase from rectal glands of *Squalus acanthias*. *Methods Enzymol* 156:43–46, 1988